

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
29 March 2001 (29.03.2001)

PCT

(10) International Publication Number
WO 01/21823 A2

(51) International Patent Classification⁷: C12N 15/86

(74) Agents: ELSY, David et al.; Withers & Rogers, Goldings House, 2 Hays Lane, London SE1 2HW (GB).

(21) International Application Number: PCT/GB00/03645

(22) International Filing Date:
22 September 2000 (22.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9922283.8 22 September 1999 (22.09.1999) GB

(71) Applicant (for all designated States except US): **THE MATHILDA & TERENCE KENNEDY INSTITUTE OF RHEUMATOLOGY** [GB/GB]; 1 Aspenlea Road, Hammersmith, London W6 8LH (GB).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **FELDMAN, Marc** [AU/GB]; 2 Church Road, London N6 4QT (GB). **FOXWELL, Brian, Maurice, John** [GB/GB]; 23 Lansdowne Road, Hownslow, Middlesex TW3 1LQ (GB).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— Without international search report and to be republished upon receipt of that report.

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ADENOVIRAL VECTORS

(57) Abstract: The invention relates to adenoviral vectors comprising a mammalian regulatory region such as a cytokine regulatory region and a reporter gene operatively linked to the cytokine promoter. Preferably, the cytokine promoter is a TNF α promoter or an IL-10 promoter. The application also discloses methods of studying cytokine promoter activity and to cells containing the adenoviral vectors.

WO 01/21823 A2

ADENOVIRAL VECTORS

The invention relates to adenoviral vectors, and in particular to adenoviral vectors comprising a mammalian regulatory region, such as a promoter, and a reporter gene operatively linked to the cytokine promoter. The invention also relates to cells containing the adenoviral vectors and to methods of using the adenoviral vectors.

Adenoviral vectors which are capable of carrying foreign DNA into cells are known in the art.

Adenoviruses exist as non-enveloped double-stranded DNA viruses. The adenovirus enters cells by a receptor-mediated endocytosis pathway. In the initial virus-receptor interaction, the adenovirus binds specific receptors present on the cell surface via fibres on its outer surface. Following attachment, the receptors with bound adenovirus cluster in coated pits, the virus is internalised within a clathrin-coated vesicle and, subsequently, into an endosomal vesicle, known as a receptosome or endosome. The adenovirus is ultimately transported to the nucleus where it directs synthesis of new nucleic acid and hence virus.

Adenovirus has been used as means of transporting DNA into cells. There are two means by which such transfer has been affected. Firstly, the adenovirus has been employed to facilitate the transfer of non-viral DNA either linked to the surface of the adenovirus or where the molecule is internalised, taken along within the adenoviral receptor-endosome complex. Secondly, the adenovirus has been employed to transfer non-viral molecules which are packaged within the adenovirus either in place of, or in addition to normal adenoviral genome. All systems have been used to take in exogenous nucleic acid placed within the double-stranded DNA genome of the virus. Such exogenous DNA has been placed under the control of a promoter to allow the exogenous nucleic acid to be transcribed.

The inventors have now produced adenoviral vectors comprising a mammalian regulatory sequence such as a cytokine promoter. Cytokines are peptide regulators of inflammatory and immune reactions. Substances considered as cytokines include, for example, interleukins 1-18, interferons, tumour necrosis factors (TNF), transforming growth factors (TGF),

platelet-derived growth factors, chemokines and colony-stimulating factors. Some compounds were sometimes previously known by the term "lymphokine".

Cytokines act in a complex interacting network on leucocytes, vascular endothelial cells, mast cells, haemopoietic stem cells, fibroblasts and osteoclasts, controlling proliferation, differentiation and/or activation through autocrine or paracrine mechanisms. Several cytokines, for example TNF- α and IL-1 are important inflammatory mediators and have been implicated in several chronic inflammatory and autoimmune diseases such as rheumatoid arthritis. The cytokines are being increasingly studied because of their importance in the formation of inflammatory and autoimmune diseases. Because the cytokines are involved in complex interconnecting reactions it is difficult to investigate the control of regulatory regions such as promoters or enhancers associated with the cytokines. The inventors realised that a cytokine promoter could be used to control a readily detectable reporter gene and allow the promoter to be studied. Furthermore, they recognised that adenovirus is a suitable vector because it enables the promoter-reporter gene construct to be inserted into a wide variety of cells including cultured cell-lines, normal cells such as macrophages, diseased tissue and primary cell-lines. The ability to insert a construct into primary cells is of particular importance because it allows the control of the promoter to be studied within, for example, diseased cells.

The use of adenovirus also had the unexpected benefit of allowing unexpectedly high levels of regulatory region-controlled reporter gene function and behaviour to be observed that were similar to the behaviour of the endogenous gene. These levels were far in excess of levels observed with other methods of inserting a cytokine regulatory region-reporter gene construct into cells. Furthermore, it has the advantage that, for the first time, cytokine promoters, such as TNF- α promoters, have been able to be studied in monocytic cells for the first time. It is expected that the high levels of expression observed means that such promoters can be studied in a variety of cells.

TNF α promoter-luciferase vectors have been produced by Udalova *et al* (J. Biol. Chem., 1998, Vol. 273, pages 21178-21186). However, the vector containing the construct, pXP-1,

can only be used in a limited number of cell types, and with low levels of expression of the promoter.

The invention provides an adenoviral vector comprising:

- a) a mammalian regulatory region; and
- b) a reporter gene operatively linked to the cytokine regulatory region (a).

The term "mammalian regulatory region" is intended to mean a region of DNA which, in its natural state, is upstream and/or downstream of a gene which it regulates. For example, it may be the promoter for the gene encoding a human oestrogen receptor.

The regulatory region may be a promoter or an enhancer region or a fragment of a region, such as an NF- κ B binding site.

The regulatory region may regulate the expression of a cytokine gene. Preferably the cytokine promoter is an interleukin promoter such as IL-10. Preferably the IL-10 promoter has the sequence from NT 7 to NT 4046 of Genbank sequence number X78437, where NT 4023 is the transcription start site, as shown in Annex 2.

Alternatively the promoter may be derived from other genes that have application in inflammatory disease, such as IL-6 and MMP.

The cytokine promoter may also be the promoter for TNF α . More especially, the TNF α receptor is a human TNF α receptor, especially the TNF α promoter identified in Genbank Accession No. M16441. This is enclosed as Annex 1. This is also the promoter used in the Udilova paper (Supra). Most preferably the sequence used is encompassed within -1173 to +130 of the human TNF gene, where 1 is the transcriptional start site. The transcriptional start site is nucleotide 4094 in the Genbank Accession No. M16441. Hence, preferably the promoter sequence starts from 2921 of M16441.

Any suitable adenoviral vector can be utilised in the present invention. A "vector" is a molecule that serves to transfer nucleic acid of interest into a cell. Thus, the adenoviral

vector utilised in accordance with the present invention can encompass any adenoviral vector that is appropriate for the introduction of nucleic acids into eukaryotic cells and is capable of functioning as a vector as that term is understood by those of ordinary skill in the art.

The adenovirus can be any serotype of adenovirus and, preferably, is a serotype that can transduce and/or infect a human cell. The adenovirus may comprise a complete adenoviral virion consisting of a core of nucleic acid and a protein capsid. Alternatively, it may comprise a naked adenoviral genome or any other manifestation of adenovirus as described in the art which can be used to transfer a cytokine regulatory region operatively linked to a reporter gene. Alternatively the virion may contain minimal (packaging signal) adenoviral genome sequences. These "gutless" viruses are produced by methods known in the art.

The adenovirus employed for transfer of the regulatory region-reporter gene construct can be wildtype, that is replication-competent. However, it is not necessary that the genome of the employed adenovirus be intact. Indeed, the adenovirus can be rendered replication-deficient, by techniques known in the art.

The reporter gene is operatively linked to the mammalian regulatory region. By this we mean that the nucleic acid encoding the reporting gene is linked to the regulatory region in such a way that the regulatory region is capable of directing transcription of the reporter gene. The reporter gene may be any nucleic acid sequence encoding a detectable gene product. The gene product may be an untranslated RNA product, such as mRNA or antisense RNA. Such untranslated RNA may be detected by techniques known in the art such as PCR, Northern or Southern blots. Alternatively, the reporter gene may encode a polypeptide, such as protein or peptide, product. Polypeptide may be detected immunologically or by means of its biological activity. The reporter genes used may be any known in the art.

Preferably, the reporter gene is luciferase. Luciferase reporter genes are known in the art. They are usually derived from firefly (*Photinus pyralis*) or seapansy (*Renilla reniformis*). The luciferase enzyme catalyses a reaction using D-luciferin and ATP in the presence of oxygen and Mg^{2+} resulting in light emission. The luciferase reaction is quantitated using a

luminometer which measures light output. The assay may also include coenzyme A in reaction which provides a longer, sustained light reaction with greater sensitivity.

An alternative reporter gene to use is CAT (chloramphenicol acetyltransferase). This is a reporter gene which is well known in the art. CAT catalyses the transfer of the acetyl group from acetyl-CoA to the substrate chloramphenicol. The enzyme reaction can be quantitated by incubating cells or cell lysates with [¹⁴C] chloramphenicol and following product formation by physical separation with, for example, thin layer chromatography or organic extraction. Alternatively, the CAT protein can be quantitated using an enzyme-linked immunosorbent assay. Such an assay is available from, for example, Promega Corporation, Southampton, United Kingdom.

A further reporter system which may be used is *lacZ* gene from *E.coli*. This encodes the β -galactosidase enzyme. This catalyses the hydrolysis of β -galactoside sugars such as lactose. The enzymatic activity in cell extracts can be assayed with various specialised substrates, for example x-gal, which allow enzyme activity quantitation using a spectrophotometer, fluorometer or a luminometer.

The reporter gene may also be gfp (green fluorescent protein) or β -globin, which are also known in the art.

The adenoviral vector preferably includes suitable termination sequences known in the art to allow the transcription of the reporter gene to be successfully terminated. Suitably, the vector also comprises a polyadenylation sequence. Such sequences may be derived from the gene from which the regulatory region is derived or alternatively from other sources such as an SV40 late polyadenylation signal to allow secretion of the reporter gene product. The termination sequences and/or polyadenylation sequences may be operatively linked 3' to the reporter gene.

The adenoviral vector may additionally comprise, 3' to the reporter gene, a 3'-UTR (untranslated region). Preferably, the 3'-UTR is from a cytokine. Preferably, the 3'-UTR is from the human TNF gene. In particular, the 3'-UTR may be from sequence identified as

Genbank Accession No. M16441, especially the sequences encoding 1041NT to the 3' of NT6071 of M16441. The sequences referred to in the paper by Udalova *et al* (Supra). 3'-UTR regions are thought to be involved effecting the stability and translation of mRNA. It is therefore advantageous to incorporate a 3'-UTR region to enable the effect of the region to be studied.

The adenoviral vector may additionally comprise one or more sequences to enable it to be maintained or replicated within a suitable bacterial host vector, such as *Escherichia coli*. Such sequences are well known in the art and include, for example, a bacterial origin or replication such as fl ori. It may also include one or more genes to enable selection of bacteria comprising the adenoviral vector, such as ampicillin or tetracycline resistance genes known in the art.

The vector may additionally comprise a further reporter gene under the control of a constitutive or unregulated promoter such as CMV promoter. The further reporter gene will be different to the first reporter gene. It acts as an internal control, allowing variations, such as the amount of vector within each cell, to be taken into account.

A further aspect of the invention provides a method of studying mammalian regulatory region activity comprising inserting an adenoviral vector according to the invention into a cell and measuring the amount of reporter gene product produced by the mammalian regulatory region encoded by the adenoviral vector. The cell into which the vector has been inserted may be exposed to one or more inhibitors or activators of the mammalian regulatory region. Cells may or may not be activated with a stimulus such as lipopolysaccharide

Alternatively, the activity in a diseased cell, for example from rheumatoid arthritic joints, may be compared with the activity of the regulatory region within a non-diseased cell. This allows a better understanding of the diseased state to be studied.

The cell into which the adenoviral vector is inserted may be selected from normal cells, macrophages, dendritic cells, phagocytes, epithelium, endothelium, tissue or cell lines. Adenoviral vectors of the invention can be introduced into cells using the natural capability of

the viruses and centre cells and to mediate uptake of macromolecules, that is by receptor-mediator endocytosis. Alternatively, the vectors can be introduced by any other suitable means such as transfection, calcium phosphate-mediated transformation, microinjection, electroporation, osmotic shock and the like.

The invention also provides a mammalian cell, especially a human cell, containing an adenoviral vector according to the invention. The type of cell may be selected from those listed above.

The invention also relates to kits comprising vectors according to the invention. Such kits may also include instructions for the use of the vector.

The invention also provides a method of screening a compound as an enhancer or an inhibitor of a mammalian regulatory region, comprising the use of an adenoviral vector according to the invention. the vector may be placed into a suitable cell, and the effect of the compound on the amount of reporter gene product produced observed. Preferably, the compound may act directly on the regulatory region or indirectly via, for example, a cascade pathway.

Embodiments of the invention will now be described by way of example only with reference to the following figures and examples.

Figure 1: Schematic representation of TNF reporter constructs.

The TNF promoter lying before the 5'UTR of TNF is represented as a thin line. The 5' and 3'UTR are represented by hatched bars. The luciferase coding sequence is shown as a solid bar. Locations of the start codon, stop codon and TATA box are indicated for each construct. For the construct TNF promoter 5'UTR, SV40 poly(A) sequence is represented by an open bar. For the construct TNF promoter 5'3'UTR, the AU-rich element (ARE) sequences are indicated as a grey box. Both constructs are produced in vector pGL3 and pAdeasy1 for transfection and adenovirus infection experiments, respectively.

Figure 2: Activity of TNF based reporter constructs when transfected or infected into RAW 264.7 and human macrophages.

A, B - cells were either transfected (*A*) or infected with adenoviral constructs at m.o.i. 40:1 (*A, B*). Following LPS (10 ng/ml.) stimulation (4h), cells were then harvested and assayed for luciferase. *C, D* - Induction by LPS (10 ng/ml.) or Zymosan (30 µg/ml.) of luciferase activity (*C*) and TNF production (*D*), in Advp5' and Advp5'3'UTR infected human macrophages. Cells were stimulated for 4h before assay. In absence of stimuli, TNF production was undetectable. Each experiment was repeated at least three times.

Figure 3: Comparison of the kinetics of LPS-induced TNF production and luciferase expression in human macrophages.

Cells were either uninfected (*A*) or infected with Advp5' (*B*) or Advp5'3'UTR (*C*) and either untreated (*O*), or activated with 10 ng/ml. LPS (*●*). At the indicated times, cell lysates or culture supernatants were harvested to assay luciferase or TNF production respectively. In the absence of LPS, TNF production was undetectable. Figure 3(*D*) Cells were infected with Advp5' (*□*) or Advp5'3'UTR (*○*) and activated with LPS for four hours. Actinomycin D was added to stop any further mRNA synthesis and the cells incubated for a further 0, 15, 30, 45 or 60 minutes. after which time they were harvested for RPA analysis of luciferase or GAPDH mRNA. The results are shown normalised to 100% at the 0 minute point. Each experiment was repeated at least two (*D*) or three (*A-C*) times with blood from distinct donors.

Figure 4: IL-10 inhibition of reporter gene constructs requires the 3'UTR.

Cells were infected with Advp5' (*A*) or Advp5'3'UTR (*B*) and activated simultaneously with LPS (10 ng/ml.) and various concentrations of IL-10. Luciferase activities (*●*) and TNF production (*○*) were assayed after 4h LPS stimulation. Ordinates represent the percentages of luciferase activation and TNF production induced by LPS, in the absence of IL-10. Data are mean values ± S.E.M. from 4 separate experiments conducted with blood from different donors.

Figure 5: Kinetics of IL-10 inhibitory activity on TNF production and luciferase expression.

Human monocytes-derived macrophages were uninfected (A) or infected with Advp5'3'UTR (B), or Advp5' (C) (m.o.i. 40:1) and then incubated with LPS in the presence (○), or absence (●) of IL-10 (10 ng/ml.). The experiment was harvested at indicated times and TNF and luciferase assayed. Data are mean values (\pm) from duplicates and are representative of three separate experiments conducted with distinct donors.

Figure 6: Effect of IL-10 on TNF and Luciferase mRNA levels.

Human macrophages infected with either Advp5', Advp5'3'UTR or Adv0 were treated with IL-10 (10 ng/ml.) simultaneously or 24h before LPS. Four hours after LPS activation, the experiment was harvested and ribonuclease protection assays were performed to quantify luciferase or TNF/GAPDH mRNA ratios. These are plotted as percentage of the maximum value in presence of LPS alone.

Figure 7: IL-10 can inhibit TNF expression even when added post-LPS stimulation.

Human macrophages infected with either Advp5' or Advp5'3'UTR (m.o.i. 40:1) were incubated for 4h in the presence of LPS (10 ng/ml.). IL-10 (10 ng/ml.) was added at the given times after LPS. Ordinates represent the percentages of luciferase activation and TNF production induced by LPS in the absence of IL-10. Data are mean values (\pm) from duplicates and are representative of three separate experiments conducted with distinct donors.

Figure 8: Effect of prolonged exposure to IL-10 prior to LPS stimulation on luciferase expression.

Human macrophages infected with either Advp5' or Advp5'3'UTR were treated with IL-10 (10 ng/ml.) for the given times prior to LPS-stimulation. Four hours after LPS activation, the experiment was harvested and luciferase activities and TNF expression assayed. Data are mean values (\pm) from duplicates and are representative of three separate experiments conducted with distinct donors.

Figure 9: Prolonged exposure to IL-10 prior to LPS-stimulation results in the inhibition of luciferase expression in the absence of 3'UTR.

Infected cells (Advp5' (A), Advp5'3'UTR (B) were pre-treated for 24h with various concentrations of IL-10 before LPS exposure. The experiment was harvested after 4h LPS stimulation and luciferase activities (●) and TNF production (○) were assayed. Data are expressed as percentages of luciferase activation and TNF production induced by LPS in absence of IL-10. Data are mean values (\pm SEM) from four experiments done with different donors.

Figure 10: In murine cells, IL-10 inhibition of luciferase expression requires the 3'UTR regardless of length of exposure to the cytokine.

Infected cells (m.o.i. 40:1) were incubated in presence of LPS (10 ng/ml.) without (■), or with IL-10 (10 ng/ml.) added simultaneously (□) or 24h before LPS addition (□). After LPS stimulation (4h), mTNF production and luciferase activities were assayed. Ordinates represent the percentages of luciferase activation and TNF production induced by LPS in the absence of cytokine. The results are representative of three (RAW264.7 cells) or two (primary murine macrophages) experiments.

METHODS

Plasmids

Human *TNF* promoter (-1173bp) with 3'untranslated region of the human *TNF* gene (pGL3-*TNF*-3'UTR) or without the 3' UT region (pGL3-*TNF*) (Udalova *et al.*, 1998) and the human *IL10* promoter construct (-4080bp) (pGL3-*IL10*) were subcloned into the pAdTrack vector (He *et al.*, 1998) to generate pAdTrack-*TNF*-Luc-3'UTR, pAdTrack-*TNR*-Luc and pAdTrack-*IL10*-Luc. For both later ones, KpnI/SalI fragments containing the human *TNF* or *IL10* promoter, the luciferase reporter gene and the SV40 late poly (A) signal were derived from their pGL3 respective constructs and cloned into KpnI/SalI sites of AdTrack vector.

The 3'UTR construct was obtained by substituting a XbaI/BamHI fragment containing the SV40 late poly(A) signal in the pGL3-*TNF* plasmid for approximately 1kbp of 3'UTR amplified by PCR with corresponding primers: 3'UTR-F (XbaI):

aattctagaGGAGGACGAACATCCAAC;

3'UTR-R(BamHI):

aatGgATcCCCAGAGTTGGAAATTC. The KpnI/SalI fragments were subsequently cloned into the pAdTrack vector.

Adenoviral vectors and their propagation

The pAdEasy-1 adenoviral plasmid was provided by B. Vogelstein (The Howard Hughes Medical Institute, Baltimore, MD) and disclosed in He *et al.* Recombinant viruses were generated in BJ5183 bacterial cells transformed by the heat-shock method with 1µg of linearised modified AdTrack constructs and 100ng of replication-deficient adenoviral vector pAdEasy-1. Positive recombinant clones were selected through their resistance to kanamycin. Following selection DNA extracted was used for virus propagation in the 293 human embryonic kidney cells. Viruses were purified by ultracentrifugation through two cesium chloride gradients as described in He *et al.*, 1998. Titres of viral stocks were determined by plaque assay in 293 cells after exposure for 1 hour in serum free DMEM medium (Gibco BRL) and subsequently overlaid with 1.6% agarose/2xDMEM with 4% FCS mixture v/v1:1 and incubated for 10-14 days (He *et al.*, 1998).

Cells

Mononuclear cells were isolated from single donor plateletpheresis residues by Ficoll-Hypaque centrifugation proceeding monocyte separation in a Beckman JE6 elutriator (High Wycombe, UK). Monocyte purity was assessed by flow cytometry with CD14 and was approx. 80% (Williams *et al.*, 1996).

The elutriated human monocytes were incubated at 1×10^6 /ml in RPMI 1640 with 25 mM HEPES and 2 mM L-glutamine supplemented with 10% (v/v) heat-inactivated FBS and 10 U/ml penicillin/streptavidin. To optimize infection, purified human monocytes were pretreated with M-CSF at 100ng/ml (Genetics Institute, Boston, MA) for 48h to allow up-regulation of integrin $\alpha_v\beta_5$, which has previously been shown to be essential for adenovirus infection of monocytes (Huang *et al.*, 1995).

RAW 264.7 mouse macrophages were maintained in DMEM supplemented with 10% FCS and 10 U/ml penicillin/streptavidin.

Transfection and Infection

0.5×10^6 RAW 264.7 cells/well were plated in growth medium (6 wells/plate). The following day, cells were transfected with 100 ng of DNA by using Superfect (Qiagen, Germany). For infection, these cells were plated at 0.1×10^6 /well (48 wells/plate). After M-CSF treatment human macrophages were replated at 0.2×10^6 /well (96 wells/plate). RAW 264.7 and M-CSF treated macrophages were then exposed to virus for 1h in serum free medium followed by washing and reculturing in growth medium with 2% FCS for 24h. For Adv *TNF*-Luc and Adv *TNF*-Luc-3'UTR, RAW 264.7 and M-CSF-treated macrophages were infected with a multiplicity of infection (MOI) of 40:1. Infected or transfected cells were then stimulated with LPS (10 ng/ml) for 4h. Infection of M-CSF treated macrophages by Adv *IL10*-Luc was efficient at MOI of 80:1 and cells were stimulated for 18h with LPS (*Salmonella typhimorium*, Sigma, Poole, UK.).

Luciferase assay

After LPS stimulation, cells were washed once in PBS and lysed with 100 µl of CAT lysis buffer (0.65% (v/v) of NP40, 10 mM of Tris-HCL pH 8, 0.1 mM EDTA pH8, 150 mM NaCl). 50 µl of cell lysate was transferred into the well of a luminometer cuvette strip and Luciferase Assay Buffer (220 µl) added. Luciferase activity was measured with a Labsystem luminometer by dispensing 30 µl luciferin (1.5 mM, Sigma) per assay point.

Measurement of hTNF and mTNF production.

TNF levels were measured in cell supernatants by sandwich enzyme-linked immunosorbent assay (ELISA). A polyclonal rabbit anti-mouse TNF antibody for coating and the same biotinylated polyclonal antibody were used to detect mTNF. ELISA for hTNF was performed as previously reported (Engelberts, I., *et al.* 1991, *Lymphokine Cytokine Res.* 1-2, 69).

Ribonuclease protection assay (RPA).

After M-CSF treatment, cells were plated at 2×10^6 /w in 12-well plate and infected as described above. In Advp5' and Advp5'3'UTR infected cells Luciferase and GAPDH mRNAs were detected by ribonuclease protection assay by using luciferase and GAPDH riboprobes, respectively. In parallel, TNF and GAPDH mRNAs were detected in Adv0-infected cells. Riboprobe vectors were constructed as follows. A 352-bp *HincII-XbaI* luciferase fragment was cloned from pGL3c (Promega) into pBluescript KS that had been digested with *EcoRV* and *XbaI*. A 268-bp TNF gene fragment was amplified by PCR from human genomic DNA, and subcloned into *SpeI* site of pBluescript KS⁺ (kindly provided by Dr A Clark, Kennedy Institute, London, UK.) that had been digested with *SpeI*. Riboprobe template constructs were linearized by appropriate restriction and purified by phenol-chloroform extraction and ethanol precipitation. Luciferase and GAPDH riboprobes were synthesized using T7 polymerase and TNF riboprobe by using T3 polymerase (Boehringer Mannheim) in the presence of 50 µCi of [α -³²P]UTP (800 mCi/mmol; Amersham). The final concentration of unlabelled UTP in *in vitro* transcription reactions was 12 µM, except in the case of Luciferase where it was 2.4 µM. Ribonuclease protection assays were carried out using the Direct

Protect kit (Ambion). Under the conditions of hybridization DNA-RNA heteroduplexes were not detected. Protected RNA fragments were resolved by electrophoresis on denaturing 6% polyacrylamide gels and were visualized and quantified by phosphorimaging (Fuji FLA 2000) and autoradiography. Each experiment was performed twice, and serial dilutions of lysates were used to check that quantitations were within the linear range of the assay.

RESULTS

Comparison of TNF reporter gene activity when delivered by adenoviral infection as opposed to standard DNA transfection.

The TNF gene reporter constructs used in this study are schematically illustrated in Figure 1. The 5'promoter and 5'promoter-3'UTR constructs, were incorporated into the pGL3 vector for transient transfection experiments (using Lipofectin), or recombinant adenoviruses (reporter viruses, Advp5' and Advp5'3'UTR respectively) for infection studies. Initial studies compared the response of the reporter gene to LPS when delivered by transfection, with infection. The RAW 264.7 murine macrophage cell line was used for these studies, as our attempts to transfect primary human monocytes or macrophages have proved unsuccessful. Lipofection was the transfection method of choice as, in our hands, this had been found to be the most efficient for the RAW 264.7 cells (results not shown). LPS stimulation of transfected cells gave a modest absolute signal and resulted in an approximate 20-fold response by both 5' and 5'3' constructs. This level of response was not dissimilar to other reported studies with the same type of construct and technique in this cell line (Hacker *et al.* (1999) *Embo J.* **24**, 6973). For comparison, both RAW 264.7 cells and primary human macrophages were infected with virus at m.o.i. 40:1, as previous studies had indicated that this concentration resulted in the successful infection of >90% of cells ((13, data not shown). In contrast to the transfection study, RAW 264.7 cells, infected with reporter adenovirus, gave LPS responses of 60-70 fold and much higher absolute values of luciferase activity (Figure 2A). LPS activation of the reporter gene in human macrophages infected with the reporter viruses resulted in 166 and 115 fold stimulation of the 5' and 5'3'UTR constructs respectively (Figure 2B). The strength of response varied from donor to donor and it has not been uncommon to obtain activations in excess of 250 fold. The greater response of human compared to murine cells has been a consistent finding. One possible reason for this is that the reporter gene constructs are based on the human sequence. The reporter gene also responded equally well to the yeast product, zymosan, an alternative stimulus of TNF production with a similar potency to LPS (Figure 2C, 2D). A consistent finding in all systems, regardless of the means of gene delivery or stimulus, was the lower absolute response of constructs containing the 3'UTR. These data would support the view, obtained

from previous studies only in murine macrophage cell lines, that the 3'UTR is generally suppressive to TNF expression (Han (1991) *J. Immunol.* 6, 1843). The powerful inducible response of the TNF gene is its notable characteristic. Therefore, it was interesting to find that the virally delivered reporter gene gave responses (activation in excess of 100 fold) that are much more comparable to the endogenous TNF gene than the transfected constructs (activation of 10 fold).

Infection

Cell type	Virus	Luciferase activity		Fold activation
		No LPS	With LPS	
RAW 264.7	Adv <i>TNF</i> -Luc	3 ± 2	216 ± 206	75 ± 34 (N=6)
	Adv <i>TNF</i> -Luc -3'UTR	0.8 ± 0.3	49 ± 60	51 ± 47 (N=6)
M-CSF treated human macrophage	Adv <i>TNF</i> -Luc	30 ± 26	4241 ± 1869	252 ± 165 (n=8)
	Adv <i>TNF</i> -Luc-3'UTR	7.5 ± 8.7	1008 ± 660	150 ± 86 (n=8)
	Adv <i>IL10</i> -Luc	1.8 ± 2	220 ± 96	159 ± 100 (n=5)

Transfection

Cell type	DNA	Luciferase activity		Fold activation
		No LPS	With LPS	
RAW 264.7	pGL3- <i>TNF</i>	2 ± 1	19 ± 7	10 ± 1 (n≥3)
	pGL3- <i>TNF</i> -3'UTR	0.17	2.5	15 (n≥3)

Kinetic studies were also performed to compare the behaviour of the reporter genes with the endogenous gene in human macrophages. In response to LPS, TNF production reached a maximum four hours after stimulation and thereafter, decreased slowly with a half-life of approximately 18.5 hours (Figure 3A). The kinetics of luciferase activity from Advp5' or Advp5'3'UTR infected LPS-stimulated human macrophages followed a similar profile to TNF, with maximum expression at 4 hours and apparent half-lives estimated to be 23.5 and 12 hours respectively (Figure 3B, 3C). The shorter half-life of luciferase, when under the addition control of the 3'UTR, might be expected from the overall destabilising effect of this element on TNF mRNA (Hacker, Supra; Kruys, *et al.* (1993) *J. Exp. Med.* 5, 1383). To confirm this, studies on luciferase mRNA stability were performed that showed the presence

of the 3'UTR does indeed increase the rate of decay of luciferase mRNA (Figure 3D). In the presence of the 3'UTR the mRNA had a half life of 56 minutes, however, in the absence of this element, there was little decay (<15%) within the same time frame. One might have expected that the half-life of the endogenous TNF protein would have been comparable to the 5'3'UTR rather than the 5' construct. However, these data do not take into account potential differences in the biological half-lives of the TNF and luciferase proteins and one can only assume that the half-life of the endogenous TNF would be longer in the absence of the 3'UTR in humans, as shown previously in mouse cells with deletions of the 3'UTR on the AU-rich region of the 3'UTR.

In primary human macrophages, IL-10 requires the 3'UTR to inhibit TNF production.

The effectiveness of adenovirus delivery of TNF-based reporter gene constructs to primary human macrophages led us to investigate what role the 5' and 3' regions may play in the IL-10 inhibition of TNF expression. Adeno-reporter virus-infected cells were simultaneously treated with LPS and various concentrations of IL-10 for 4 hours, after which TNF production and luciferase activities were assayed (Figure 4). IL-10 inhibited TNF expression to a maximum of 80% at 10 ng/ml. (Figure 4A, 4B). However, the response of the two reporter constructs was quite distinct. The 5' construct was only weakly inhibited by IL-10, approximately 10%, whereas the 5'3'UTR construct showed a dose response profile similar to the endogenous TNF, although the maximum inhibition attained was less, 60% at 10 ng/ml. The IC_{50} for IL-10 on TNF protein was 0.2-0.3 ng/ml., compared with 2-3 ng/ml. for the reporter gene. However, if the concentration of half-maximal inhibition is calculated, then the activity of IL-10 is similar for the endogenous gene (~0.1 ng/ml.) and the 5'3'UTR construct (0.2-0.3 ng/ml.). This suggests that aspects of the inhibitory activity of IL-10 on the reporter gene and the endogenous gene are similar. The kinetics of the IL-10 inhibition of TNF and luciferase expression was also compared using the optimal concentration of 10 ng/ml. (Figure 5). Over the two to twenty-four hour period post-stimulation, the expression of TNF and the 5'3'UTR construct gave very similar profiles with very little response detected in the presence of IL-10 (Figure 5A, 5B). The effect of IL-10 on the 5' construct was, again, much weaker, with no significant inhibition over the time course (Figure 5C). These data would suggest that the major inhibitory effect of IL-10 is mediated via the 3'UTR and that

there appears to be little effect on the transcription of the gene when IL-10 is administered at the same time as LPS. To gain a further insight into the mechanism of IL-10 activity, luciferase mRNA levels were analysed by RPA. As shown in Figure 6, simultaneous IL-10 addition caused a marked reduction of luciferase mRNA from the 5'3'UTR construct, whereas, there was only a marginal effect on mRNA from the 5' construct. These data indicates that IL-10 causes a potential destabilization/enhanced destruction of mRNA via the 3'UTR. Data obtained with TNF mRNA showed identical results to the 5'3'UTR construct (Figure 6).

The data so far indicates that IL-10 mediates its activity on the post-transcriptional mechanism associated with TNF production. If this is so, IL-10 should still be able to inhibit TNF production, at least for a period, if added after LPS. Reporter virus infected macrophages were stimulated with LPS and IL-10 was added for periods of up to 2 hours post-activation; as before the experiment was harvested at 4 hours for assay. As expected, IL-10 had little effect on the activity of the 5' construct, regardless of when it was added (Figure 7). The inhibitory effect of IL-10 on TNF and the expression of the 5'3'UTR construct was maintained, even if IL-10 was added 1 hour after LPS activation but was greatly reduced if the cytokine was added 2 hours post-activation (Figure 7). These data would suggest that IL-10 does not inhibit the early events that are involved in TNF production (e.g. transcription) and instead, targets later events (e.g. post-transcriptional). This agrees with the hypothesis that post-transcriptional control mediated via the 3'UTR is the target of IL-10.

Pre-incubation of human macrophages with IL-10 before LPS stimulation reveals a second mechanism of inhibiting TNF production through the 5' promoter.

As time of exposure to IL-10 could obviously have a bearing on its function, this study was extended to investigate the effect of adding IL-10 **before** LPS for periods up to 24 hours (Figure 8). Pre-incubating macrophages with IL-10 for 12 hours produced a modest increase in the inhibition of endogenous TNF production or luciferase activity from the 5'3'UTR reporter gene when compared with the effect of simultaneous addition of LPS and IL-10 (Figure 8). Further periods of pre-incubation up to 24 hours did not elicit any major

additional effect. However, pre-exposure of cells to IL-10 had a profound effect on the expression of the 5' reporter. Pre-incubation of macrophages with IL-10 for twelve hours before LPS stimulus resulted in 50% inhibition of the 5' construct that increased to nearly 70% when the pre-incubation period was extended to 24 hours (Figure 8). This was compared to the 80% inhibition of endogenous TNF production. The effect of pre-incubating macrophages for 24 hours with different concentrations of IL-10 was also examined. As shown in Figure 9, LPS-induced TNF protein expression was inhibited to a maximum of 90% (10 ng/ml. IL-10) with an IC50 of ~0.1 ng/ml. regardless of which reporter construct had been infected into the macrophages. IL-10 also inhibited the expression of the 5'3'UTR reporter to an identical degree to the endogenous gene (Figure 9B). However, in contrast to data in Figure 4A, pre-incubation for 24 hours with IL-10 now produced a dose-dependent inhibition of the 5'UTR construct that showed a maximum inhibition of 60% at 10 ng/ml. (Figure 9A). The IC50 for IL-10 on the 5' construct was 5 ng/ml. but this reduced to 0.5 ng/ml. if the half-maximal inhibition was again calculated. These data would indicate that in addition to post-transcriptional regulation of the TNF gene, IL-10 can also inhibit transcription of the TNF gene if cells are exposed to this inhibitory factor for a sufficient period. Indeed, studies on mRNA levels showed that pre-incubation, for 24 hours, with IL-10 resulted in a decrease in luciferase mRNA regardless of the presence of the 3'UTR (Figure 6).

IL-10 cannot inhibit TNF gene by the 5' promoter in primary murine macrophages and cell lines.

To the inventors' knowledge, the potential dual mechanism of IL-10 inhibitory activity on TNF expression has not been previously described. However, the type of reporter gene study described here is also novel. We therefore applied the adeno-reporter virus approach to murine macrophage cell lines and murine peritoneal derived macrophages systems that are more commonly used to investigate IL-10 function. We have previously shown that the murine macrophage cell line RAW 264.7 would be infected by adenovirus (Figure 2), and we have found that murine peritoneal derived macrophages were also permissive to virus infection (results not shown). We observed that a m.o.i. 40 of adenovirus was sufficient to infect murine primary cells and that LPS would activate both the 5' and 5'3'UTR constructs

(results not shown). Murine cells were infected with reporter viruses and were either pre-incubated with IL-10 for 24 hours or given the cytokine simultaneously with LPS (Figure 10). We had previously ascertained that 10 ng/ml. IL-10 was an optimal concentration for the inhibition of TNF production by the murine cells (23). For simplicity the data with IL-10 from each study are shown as a comparison with the LPS only control that is given as 100% (Figure 10). In RAW 264.7 cells, IL-10 inhibited TNF production and 5'3' luciferase construct to a similar degree when added with LPS. However, there was no inhibition of the 5' construct even when RAW 264.7 cells were pre-incubated with IL-10. Also, pre-exposure to the cytokine reduced the inhibition of luciferase expression by the 5'3' construct although the inhibition of endogenous TNF was maintained. The study was repeated on primary murine macrophages since RAW 264.7 are transformed and this could affect the mechanisms of IL-10 action. IL-10 produced a profound inhibition of TNF production by primary murine macrophages regardless of length of treatment with cytokine. However, the effect on the expression of 5'3'UTR was only moderate, achieving a 30% inhibition. Again, this was not affected by pre-incubation with IL-10. There was no effect on the expression of the 5' construct under any of these circumstances. A trivial reason for the failure to demonstrate any effect on 5' construct, at least on the murine cells, was that these studies were performed with human rather than murine IL-10. However, repetition of these studies with the murine IL-10 gave similar results (results not shown).

DISCUSSION

The results show that viral transfer allows improved reporter gene induction (60 to 80 fold) in cell lines and even more so in primary cells (approximately 200 fold) following LPS stimulation. This result is unexpected and demonstrates that use of an adenoviral construct has unexpected advantages over the prior art.

The constructs have also been demonstrated to be able to be used to study regulatory region, such as cytokine, activity with primary macrophages which has previously been difficult to undertake.

REFERENCES

Udalova, I.A., Knight, J.C., Vidal, V., Nedospasov, S.A. & Kwiatkowski, D. Complex NF-kappaB interactions at the distal tumor necrosis factor promoter region in human monocytes. *J Biol Chem* **273**, 21178-86 (1998).

He, T.C., *et al.* A simplified system for generating recombinant adenoviruses, *Proc Natl Acad Sci USA* **95**, 2509-14 (1998).

Huang S., *J. Virol.*, 69(4), 2257-63 (1995).

Williams, L.M., Gibbons, D.L., Gearing, A., Maini, R.N., Feldmann, M. & Brennan, F.M. Paradoxical effects of a synthetic metalloproteinase inhibitor that blocks both p55 and p75 TNF receptor shedding and TNF α processing in RA synovial membrane cell cultures. *J Clin Invest* **97**, 2833-41 (1996).

ANNEX 1

LOCUS (LOC): HUMTNFAB GenBank (R)
 GenBank ACC. NO. (GBN): M16441
 CAS REGISTRY NO. (RN): 140752-05-2
 SEQUENCE LENGTH (SQL): 7112
 MOLECULE TYPE (CI): DNA; linear
 DIVISION CODE (CI): Primates
 DATE (DATE): 14 Jan 1995
 DEFINITION (DEF): Human tumor necrosis factor and lymphotoxin genes, complete cds.
 KEYWORDS (ST): lymphotoxin; tumor necrosis factor
 SOURCE: Human placenta DNA, clone pTNF186.
 ORGANISM (ORGN): Homo sapiens
 Eukaryotae; mitochondrial eukaryotes; Metazoa;
 Chordata; Vertebrata; Eutheria; Primates;
 Catarrhini; Hominidae; Homo
 NUCLEIC ACID COUNT (NA): 1676 a 2005 c 1865 g 1566 t
 ORIGIN: 1 bp upstream of EcoRI site; chromosome 6p21.3.
 COMMENT: Draft entry and computer-readable sequence for [1]
 kindly submitted by C.V.Jongeneel, 02-OCT-1988.
 REFERENCE: 1 (bases 1 to 7112)
 AUTHOR (AU): Nedospasov,S.A.; Shakhov,A.N.; Turetskaya,R.L.;
 Mett,V.A.; Azizov,M.M.; Georgiev,G.P.; Korobko,
 V.G.; Dobrynin,V.N.; Filippov,S.A.; Bystrov,N.S.;
 Boldyreva,E.F.; Chuvpilo,S.A.; Chumakov,A.M.;
 Shingarova,L.N.; Ovchinnikov,Y.A.
 TITLE (TI): Tandem arrangement of genes coding for tumor
 necrosis factor (TNF-alpha) and lymphotoxin
 (TNF-beta) in the human genome
 JOURNAL (SO): Cold Spring Harb. Symp. Quant. Biol., 51 Pt 1,
 611-624 (1986)
 OTHER SOURCE (OS): CA 107:110246

FEATURES (FEAT):

Feature Key	Location	Qualifier
source	1..7112	/organism="Homo sapiens"
prim-transcript	819..2855	/map="6p21.3"
intron	980..1266	/note="lymphotoxin mRNA and introns"
CDS	join(1276..1374, 1461..1566,1814..2226)	/note="lymphotoxin intron A"
		/codon-start=1
		/db-xref="PID:g339740"
		/translation="MTPPERLFLPRVCGTTLHLL LLGLLLVLLPGAQGLPGVGLTPSA AQTAHQHPKMH LAHSTLKPAAHLIGDPSKQNSLL WRANTDRAFLQDGFSLSNNSLLVP TSGIYFVYSQVVFSGKAYSPKATSSPLYLAHEVQ

gene 1276..1374
 exon <1276..1374

intron 1375..1460
 exon 1461..1566
 intron 1567..1813
 exon 1814..>2226

prim-transcript 4095..6859
 exon <4275..4460

gene 4275..4460
 CDS join(4275..4460,
 5067..5112,5300..5347,
 5649..6070)

intron 4461..5066
 exon 5067..5112
 intron 5113..5299
 exon 5300..5347
 intron 5348..5648

LFSSQYPFHVPLLSSQKMVYPGLQ
 EPWLHSMYHGAAAFQLTQGDQLSTHTDGIPHLVLS
 PSTVFFGAFAL"
 /gene="TNFB"
 /gene="TNFB"
 /note="lymphotoxin, (first
 expressed exon); G00-120-442"
 /number=2
 /note="lymphotoxin intron B"
 /number=3
 /note="lymphotoxin intron C"
 /note="lymphotoxin"
 /number=4
 /note="TNF mRNA and introns"
 /gene="TNFA"
 /note="tumor necrosis factor;
 G00-120-441"
 /number=1
 /gene="TNFA"
 /note="tumor necrosis factor"

/codon-start=1
 /db-xref="PID:g339741"
 /translation="MSTESMIRDVELAEALPKK
 TGGPQGSRRCLFLSLFSFLIVAGA
 TTLFCLLHFGVIGPQREFFPRDLSLISPLAQAVR
 SSSRTPSDKPVAVHVANPQAEQQL
 QWLNRRANALLANGVELRDNQLVVPSEGLYLIYS
 QVLFKGQGCPSSTHVLTTHTISRIA
 VSYQTKVNLLSAIKSPQRETPEGAEAKPWYEPI
 YLGGVFQLEKGDRLSAEINRPDYL
 DFAESGQVYFGIIAL"
 /note="TNF intron A"
 /note="TNF intron B"
 /number=3
 /note="TNF intron C"

SEQUENCE (SEQ):

```

1 gaattctcga aacttccttt gtagaaaact ttggaaggtg tctgccacat tgatcctgga
61 atgtgtgttt atttgggggtt atataaatct gttctgtgga agccacctga agtcaggaag
121 agatggaggg catccttcag gagtgagatg agacctcatc atacttgact gtccagcatc
181 atctctgagt gaggggacca aaaaatttat cttccaaact aggacacttt caagagtggga
241 aggggggatcc attaatatatt tcacctggac aagaggcaaa caccagaatg tccccgatga
301 aggggatata taatggacct tcttgatgtg aaacctgcca gatgggctgg aaagtccgta
361 tactgggaca agtatgattt gagttgtttg ggacaaggac aggggtacaa gagaaggaaa
421 tgggcaaaga gagaagcctg tactcagcca aggggtgcaga gatgttatat atgattgctc
481 ttcagggaac cgggcctcca gctcacaccc cagctgctca accacctcct ctctgaattg
541 actgtccctt ctttggaact ctaggcctga cccactccc tggccctccc agcccacgat
601 tcccctgacc cgactccctt tcccagaact cagtcgcctg aacccccagc ctgtggttct
661 ctccctaggcc tcagcctttc ctgcctttga ctgaaacagc agtatcttct aagccctggg
721 ggcttccccg ggccccagcc ccgacctaga acccgcccgc tgccctgcac gctgccactg
781 ccgcttcctc tataaaggga cctgagcgtc cgggcccagg ggctccgcac agcaggtgag
841 gctctcctgc cccatctcct tgggctgccc gtgcttctgt ctttgacta ccgcccagca
901 gtgtcctgce ctctgcctgg gcctcggtcc ctccctgcacc tgctgcctgg atccccggcc
961 tgccctgggce tgggccttgg tgggtttggt tttggtttcc ttctctgtct ctgactctcc
1021 atctgtcagt ctcatgtct ctgtcacaca ttctctgttt ctgccatgat tccctctctgt
1081 tcccttccctg tctctctctg tctccctctg ctccacttgg gggttctctg actgcatctt
1141 gtcccttctct ctgtcgatct ctctctcggg ggtcgggggg tgctgtctcc cagggcggga
1201 ggtctgtctt ccgcgcgctg ccccgccccg ctcaactgtct ctctctctct ctctcttctt
1261 ctgcagggtt tccccatgac accacctgaa cgtctcttcc tcccaagggt gtgtggcacc

```

1321	accctacacc	tctctcttct	ggggctgctg	ctgggttctgc	tgcttggggc	ccaggtgagg
1381	cagcaggaga	atgggggctg	ctgggggtgc	tcagccaaac	cttgagccct	agagccccc
1441	tcaactctgt	tctcccctag	gggctccctg	gtgttggcct	cacaccttca	gctgccccaga
1501	ctgcccgtca	gcaccccag	atgcatcttg	cccacagcac	cctcaaacct	gctgctcacc
1561	tcattggtaa	acatccacct	gacctcccag	acatgtcccc	accagctctc	ctcctacccc
1621	tgcttcagga	acccaagcat	ccacccctct	cccccactt	cccccacgct	aaaaaaaaaca
1681	gagggagccc	actcctatgc	ctccccctgc	catccccag	gaactcagtt	gttcagtgcc
1741	cacttctctca	gggattgaga	cctctgatcc	agacccctga	tctcccaccc	ccatccccta
1801	tggtctcttcc	taggagaccc	cagcaagcag	aactcactgc	tctggagagc	aaacacggac
1861	cgtgccttcc	tccaggatgg	tttctccttg	agcaacaatt	ctctcctggg	ccccaccagt
1921	ggcatctact	tcgtctactc	ccaggtggtc	ttctctggga	aagcctactc	tcccaaggcc
1981	acctcctccc	cactctacct	ggcccatgag	gtccagctct	tctcctccca	gtaccccttc
2041	catgtgcctc	tcctcagctc	ccagaagatg	gtgtatccag	ggctgcagga	accctggctg
2101	cactcgatgt	accacggggc	tgcttccag	ctcaccagg	gagaccagct	atccaccac
2161	acagatggca	tccccacct	agtcctcagc	cctagtactg	tcttctttgg	agccttcgct
2221	ctgtagaact	tggaataatc	cagaaagaaa	aaataattga	tttcaagacc	ttctcccat
2281	tctgcctcca	ttctgaccat	ttcaggggtc	gtcaccacct	ctcctttggc	cattccaaca
2341	gctcaagtct	tccctgatca	agtcaccgga	gctttcaaag	aaggaattct	aggcatccca
2401	ggggaccaca	cctccctgaa	ccatccctga	tgtctgtctg	gctgaggatt	tcaagcctgc
2461	ctaggaattc	ccagcccaaa	gctgttggtc	ttgtccacca	gctaggtggg	gcctagatcc
2521	acacacagag	gaagagcagg	cacatggagg	agcttggggg	atgactagag	gcagggaggg
2581	gactatttat	gaaggcaaaa	aaattaaatt	atttatttat	ggaggatgga	gagaggggaa
2641	taatagaaga	acatccaagg	agaaacagag	acaggcccaa	gagatgaaga	gtgagagggc
2701	atgcgcacaa	ggctgaccaa	gagagaaaga	agtaggcag	agggatcaca	agggcccgaga
2761	agggcaggaa	aggctctgaa	agccagctgc	cgaccagagc	cccacacgga	ggcatctgca
2821	ccctcgatga	agcccaataa	acctcttttc	tctgaaatgc	tgtctgcttg	tgtgtgtgtg
2881	tctgggagtg	agaacttccc	agtctatcta	aggaatggag	ggagggacag	agggctcaaa
2941	gggagcaaga	gctgtgggga	gaacaaaagg	ataagggctc	agagagcttc	agggatatgt
3001	gatggactca	ccaggtgagg	ccgccagact	gctgcagggg	aagcaaagga	gaagctgaga
3061	agatgaagga	aaagtcaggg	tctggagggg	cgggggtcag	ggagctcctg	ggagatatgg
3121	ccacatgtag	cggctctgag	gaatgggtta	caggagacct	ctggggagct	gtgaccacag
3181	caatgggtag	gagaatgtcc	agggctatga	aagtcgagta	tggggacccc	cccttaacga
3241	agacagggcc	atgtagaggg	ccccaggagg	tgaagagacc	tccaggacct	ccaggtatgg
3301	aatacagggg	acgtttaaga	agatatggcc	acacactggg	gccctgagaa	gtgagagctt
3361	catgaaaaaa	atcagggacc	ccagagttcc	ttggaagcca	agactgaaac	cagcattatg
3421	agtctccggg	tcagaatgaa	agaagagggc	ctgccccagt	ggggtctgtg	aattcccggg
3481	ggtgatattca	ctccccgggg	ctgtcccagg	cttgtccctg	ctacccgcac	ccagcctttc
3541	ctgaggcctc	aagcctgcca	ccaagccccc	agctccttct	ccccgcaggg	cccaaacaca
3601	ggcctcagga	ctcaacacag	cttttccctc	caaccccggt	ttctctccct	caacggactc
3661	agctttctga	agccctctcc	agttctagtt	ctatcttttt	cctgcctcct	gtcttgaagt
3721	tagaaggaaa	cagaccacag	acctggtccc	caaaagaaat	ggaggcaata	gtttttgagg
3781	ggcatgggga	cgggggttcag	cctccagggt	cctacacaca	aatcagtcag	tggcccagaa
3841	gacccccctc	ggaatcggag	cagggaggat	ggggagtgtg	aggggtatcc	ttgatgcttg
3901	tgtgtcccca	actttccaaa	tccccgcccc	cgcgatggag	aagaaaccga	gacagaaggt
3961	gcagggccca	ctaccgcttc	ctccagatga	gctcatgggt	ttctccacca	aggaagtttt
4021	ccgctgggtg	aatgattctt	tccccgcccc	cctctcggcc	cagggacata	taaaggcagt
4081	tgttggcaca	cccagccagc	agacccctcc	tcagcaagga	cagcagagga	ccagctaaga
4141	gggagagaa	caactacaga	ccccccctga	aaacaaccct	cagacgccac	atcccctgac
4201	aagctgccag	gcagggttctc	ttcctctcac	atactgaccc	acggcttcac	cctctctccc
4261	ctggaaaagga	caccatgagc	actgaaagca	tgatccggga	cgtggagctg	gccgaggagg
4321	cgctcccca	gaagacaggg	gggccccagg	gctccaggcg	gtgcttgctc	ctcagcctct
4381	tctccttcc	gatcgtggca	ggcgccacca	cgctcttctg	cctgctgcac	tttgagtgga
4441	tcggccccca	gagggaaagag	gtgagtgcct	ggccagcctt	catccactct	cccacccaag
4501	gggaaatgga	gacgcaagag	agggagagag	atgggatggg	tgaaagatgt	gcgctgatag
4561	ggagggatgg	agagaaaaaa	acgtggagaa	agacggggat	gcagaaagag	atgtggcaag
4621	agatggggaa	gagagagaga	gaaagatgga	gagacaggat	gtctggcaca	tggaaaggtgc
4681	tcactaagt	tgtatggagt	gaatgaatga	atgaatgaat	gaacaagcag	ataataaat
4741	aagatatgga	gacagatgtg	gggtgtgaga	agagagatgg	gggaagaaac	aagtgatatg
4801	aataaagatg	gtgagacaga	aagagcggga	aatatgacag	ctaaggagag	agatggggga
4861	gataaggaga	gaagaagata	gggtgtctgg	cacacagaag	acactcaggg	aaagagctgt
4921	tgaatgcctg	gaaggtgaat	acacagatga	atggagagag	aaaaccagac	acctcagggc
4981	taagagcgca	ggccagacag	gcagccagct	gttctctcct	taagggtgac	tccctcgatg

5041 ttaaccattc tcttctctcc caacagttcc ccaggacct ctctotaatc agccctctgg
5101 cccaggcagt cagtaagtgt ctccaaacct ctttcctaata tctggggttg ggtttggggg
5161 taggggttagt accggtatgg aagcagtggg ggaaatttaa agttttggtc ttgggggagg
5221 atggatggag gtgaaagtag ggggggtattt tctaggaagt ttaaggtct cagcttttct
5281 ttttctctct cctcttcagg atcatcttct cgaaccccca gtgacaagcc tgtagcccat
5341 gttgtaggta agagctctga ggtgtgtct tggaacttgg agggctagga tttggggatt
5401 gaagcccggc tgatggtagg cagaacttgg agacaatgtg agaaggactc gctgagctca
5461 aggggaaggt ggaggaacag cacaggcctt agtgggatac tcagaacgtc atggccaggc
5521 gggatgtggg atgacagaca gagaggacag gaaccggatg tggggtgggc agagctcgag
5581 ggccaggatg tggagagtga accgacatgg ccacactgac tctcctctcc ctctctccct
5641 ccctccagca aacctcaag ctgaggggca gctccagtgg ctgaaccgcc gggccaatgc
5701 cctcctggcc aatggcgtgg agctgagaga taaccagctg gtggtgccat cagagggcct
5761 gtacctcatc tactcccagg tcctcttcaa gggccaaggc tgccccctca cccatgtgct
5821 cctcaccac accatcagcc gcctgcctt ctcctaccag accaaggtca acctcctctc
5881 tgccatcaag agccctgcc agaggagac ccagagggg gctgaggcca agccctggta
5941 tgagcccatc tatctgggag ggtcttcca cctggagaag ggtgaccgac tcagcgctga
6001 gatcaatcgg ccgactatc tcgactttgc cgagtctggg caggtctact ttgggatcat
6061 tgccctgtga ggaggacgaa catccaacct tcccaaaccg ctccccctgcc ccaatccctt
6121 tattaccccc tccttcagac accctcaacc tcttctggct caaaaagaga attgggggct
6181 tagggtcgga acccaagctt agaactttaa gcaacaagac caccacttcg aaacctggga
6241 ttcaggaatg tgtggcctgc acagtgaagt gctggcaacc actaagaatt caaactgggg
6301 cctccagaac tctctggggc ctacagcttt gatccctgac atctggaatc tggagaccag
6361 ggagcctttg gttctggcca gaatgctgca ggacttgaga agacctcacc tagaaattga
6421 cacaagtgga ccttaggcct tcctctctcc agatgtttcc agacttcctt gagacacgga
6481 gcccagccct ccccatggag ccagctccct ctatttatgt ttgcacttgt gattatttat
6541 tattttattta ttatttatatt atttacagat gaatgtattt atttgggaga ccggggtatc
6601 ctgggggacc caatgtagga gctgccttgg ctgagacatg ttttccgtga aaacggagct
6661 gaacaatagg ctgttcccat gtagccccct ggcctctgtg ccttcttttg attatgtttt
6721 ttaaaatatt tatctgatta agttgtctaa acaatgctga tttggtgacc aactgtcact
6781 cattgctgag cctctgctcc ccaggggagt tgtgtctgta atcgccctac tattcagtgg
6841 cgagaaataa agtttgctta gaaaagaaac atggtctcct tcttgggaatt aattctgcat
6901 ctgcctcttc ttgtgggtgg gaagaagctc cctaagtcct ctctccacag gctttaagat
6961 ccctcggacc cagtcccatc cttagactcc tagggccctg gagaccctac ataaacaaag
7021 cccaacagaa tattcccat ccccaggaa acaagagcct gaacctaat acctctccct
7081 cagggcattg gaatttcaa ctctgggaat tc

ANNEX 2

GENBANK.RTM. COPYRIGHT 1999

LOCUS (LOC): HSINTL10 GenBank (R)
 GenBank ACC. NO. (GBN): ***X78437***
 CAS REGISTRY NO. (RN): 156795-75-4
 SEQUENCE LENGTH (SQL): 4181
 MOLECULE TYPE (CI): DNA; linear
 DIVISION CODE (CI): Primates
 DATE (DATE): 27 Jan 1997
 DEFINITION (DEF): H.sapiens IL-10 gene.
 KEYWORDS (ST): IL-10 gene; interleukin 10
 SOURCE: human.
 ORGANISM (ORGN): Homo sapiens
 Eukaryotae; mitochondrial eukaryotes; Metazoa;
 Chordata; Vertebrata; Eutheria; Primates;
 Catarrhini;
 Hominidae; Homo

NUCLEIC ACID COUNT (NA): 1181 a 1046 c 961 g 993 t
 REFERENCE: 1 (bases 1 to 4181)
 AUTHOR (AU): Kube,D.; Platzter,C.; von Knethen,A.; Straub,H.;
 Bohlen,H.; Hafner,M.; Tesch,H.
 TITLE (TI): Isolation of the human interleukin 10 promoter.
 Characterization of the promoter activity in
 Burkitt's
 lymphoma cell lines
 JOURNAL (SO): Cytokine, 7 (1), 1-7 (1995)
 OTHER SOURCE (OS): CA 122:125051
 REFERENCE: 2 (bases 1 to 4181)
 AUTHOR (AU): Kube,D.
 TITLE (TI): Direct Submission
 JOURNAL (SO): Submitted (21-MAR-1994) D. Kube, Medizinische
 Klinik I
 der Universitaet zu Koeln, LFI Ebene 4 Raum 508,
 Joseph-Stelzmann-Str 9, 50924 Koeln, FRG
 REFERENCE: 3 (bases 1 to 4181)
 AUTHOR (AU): Kube,D.
 TITLE (TI): Direct Submission
 JOURNAL (SO): Submitted (26-APR-1995) D. Kube, Medizinische
 Klinik I
 der Universitaet zu Koeln, LFI Ebene 4 Raum 508,
 Joseph-Stelzmann-Str 9, 50924 Koeln, FRG
 REFERENCE: 4 (bases 1 to 4181)
 AUTHOR (AU): Kube,D.
 TITLE (TI): Direct Submission
 JOURNAL (SO): Submitted (25-JAN-1996) D. Kube, Medizinische
 Klinik I
 der Universitaet zu Koeln, LFI Ebene 4 Raum 508,
 Joseph-Stelzmann-Str 9, 50924 Koeln, FRG
 REFERENCE: 5 (bases 1 to 4181)
 AUTHOR (AU): Eskdale,J.; Kube,D.; Gallagher,G.
 TITLE (TI): A second polymorphic dinucleotide repeat in the 5'
 flanking region of the human IL10 gene
 JOURNAL (SO): Immunogenetics, 45 (1), 82-83 (1996)
 OTHER SOURCE (OS): CA 126:113969

FEATURES (FEAT):
 Feature Key Location Qualifier
 =====+=====+=====+
 source 1..4181 /organism="Homo sapiens"

mRNA 4023..>4181
 gene 4023..4181
 CDS 4051..>4181

/tissue-type="placenta"
 /clone-lib="pWe15 cosmid library"
 /gene="Il-10"
 /evidence=experimental
 /gene="Il-10"
 /gene="Il-10"
 /codon-start=1
 /product="interleukin-10"
 /db-xref="PID:e220327"
 /db-xref="PID:g1177469"
 /translation="MHSSALLCCLVLLTGVRASP
 GQGTQSENSCTHFPGNLPNMLRDL "

SEQUENCE (SEQ):

```

1 agatcttgta aactgtagaa tgcaccctcc aaaatctatt tgcataagca cacacacaca
61 cacacacaca cacaccccag cagttcttgc ctgcccagat tcctctgcag cttaaagtgat
121 gaaacttact gggcggagct tcctaaaaag attattaggg tctcctgggt tgggtgcct
181 ttaaaccctt ggactttacc acctcctatc tctcctatct ccttgcaaca aaggtagga
241 gaacaagaat gcagaaaaaa cgggtcctgg atgacatctg agtgccctgt ttgggcttct
301 tgatgagtga gacagaaaat aaaatacaac cccctctttt aaaagccatg cttactcagg
361 ttttccttca tttgcagcta aatacagaaa tgagagaata ttttgagca gggatggaag
421 aagagaggta ttccccctcc cacaaccttc tgatttccca gtacatcccc cactggaaaa
481 attcatttaa aatcagtata ataagcattg attagatgcc tactatgcac ctgggcttga
541 gggcaaactg gactcaggcc ttttgccctc aagaagctca cagtgtgaga gtggcatttg
601 tgtcctcttg aaattcacag gactaaattg tgcccaggct gacattctat ccatccatag
661 gtgcttgcct tctcacttcc ctctcttcat gggctcttgc cttgtaccaa aatccaaacc
721 caaatctcct cacatgtgag tgttggcatt catgtctcag acatgaccta tgggcttggg
781 acttttcccc gtggacccca gtgacttttc agatgaacag gtatcttcaa aaacttgaga
841 aataggagtc ctgtttgttg ttcttgttgc tttgtcaata taaggcacag ggtctttatt
901 caaatgttca tactatctct tgacagaaat actatgagac atattgatgg agaagccgtt
961 atctccatat gctaaatgag gacttgacc agggaacttg ccatgggttc tctccaacca
1021 cttaaattct gaaattttga atgagagtgg acagtaattt caaatcaatg gggaaagaat
1081 caaatcttca gcaaattggc tgagataatt agctacacat ttcagaacaa ataaagaagt
1141 cagatccggg ccgggcacag tggctcatgc ctgtaatctc agcactctgg gaggccaagg
1201 cgggcggtac ataaggtcag gagatcgaga ccatcctggt taacacagtg aaccctctc
1261 taataaaaaa acaaaaaaac ataaaaaac ttagccgggc gtggtgccag cgcagcttag
1321 tcccagctac tcgggacgct gaggcaggag aatggcttga actcgggagg gagagcttgc
1381 aggtgagctg agatcatgcc actgcactcc agcctgggca acagagcgag actctgtctc
1441 aaaaaaaaaa aagaagtcag atcctaacct caaccctatt taacagatta tagatgaaag
1501 aaaggtacaa atggctttta catacctccc ttctccctga cattttgtat gtgtgtgtgt
1561 gtgtattttac acacacatct catataagga aattgaaggg aggtgcctg catccctgag
1621 tcactctccc tctccttctg aatgcttacc tgtgccaga ccacctcctt cctctgcac
1681 cctccaggct tacagggcac tcttctatgc ccatcccaag tatagctgaa ccttccaagg
1741 gccagacttg gtgctaagta ccaagtacgc aaagattaat aaaacaatgt cctgtttcag
1801 ggagctcaaa gctgattcgg cagggcatgg tgtgtacatg aatgataacc acgtagggtt
1861 gcagggtttcc tagtgaggta agcacaaggc aagatgggaa acaaaggag gagggttca
1921 cagcctcacc cagagtccag aaccctggc ctgcctgggt cccatgctga gtccacttct
1981 ggaacaccca gctcagagag ggggttagac ctgcaggcta acacagacac agcccagaaa
2041 acccaggagc cgagggggaa ggagaaagg gcaagaagg gaaacccagg tccctgtccc
2101 ctctctctct ctctctggca gcagaactca gacagaaccc ttaagccagt ctaagtctgg
2161 caggaccagt aagttctgag ttagctccat actagtttct agcaggctct ttctcacttc
2221 ctgattctta ggtttctaca ttgacactcc ctgaagagtt gggaagagac accacagtcc
2281 cctgaccctg atccataggt cacacagcag gcatccacag ggtgggctg ggccctctca
2341 tccctccctc cactcactt cacgtggct cagcgtcag aacctgctgc cactggagt gtcccattgc
2401 gagtgaacct ctctagtcca ccaagctcag aacctgctgc cactggagt gtcccattgc
2461 tgatgcagaa aggtgaagaa ctagcagaac actggaaatg cctccatct ggggtccatg
2521 ctacttaatg ctccctggca ggcaggagga caggtgctat tccctgttgg gacagatgaa
2581 aaacagacac agggaggatg agtgatttgc cctgactata gagtggcagg gccaaggcag
2641 agcccaggcc tctgcacct aggtcagtg tctcccagt tacagtctaa actggaatgc
2701 aggcaaaagg cctgtggaag gggaaaggta aggtcaatc aaaggatccc cagagacttt
2761 ccagatatct gaagaagtcc gtaggtcact gccccggtcc tccccaggt tccccaacac
2821 tctctgcccgc aaccctaact gctccccctta ccttctacac acacacacac acacacacac
2881 acacacacac acacacacaa atccaagaca aactactaa ggcttctttg ggaaggggaa
2941 gtaggggatg gtaagaggaa agtaagggac ctccatcca gctccatgg aatcctgact
3001 tcttttctct gttatttcaa cttcttccac cccatctttt aaactttaga ctccagccac

```

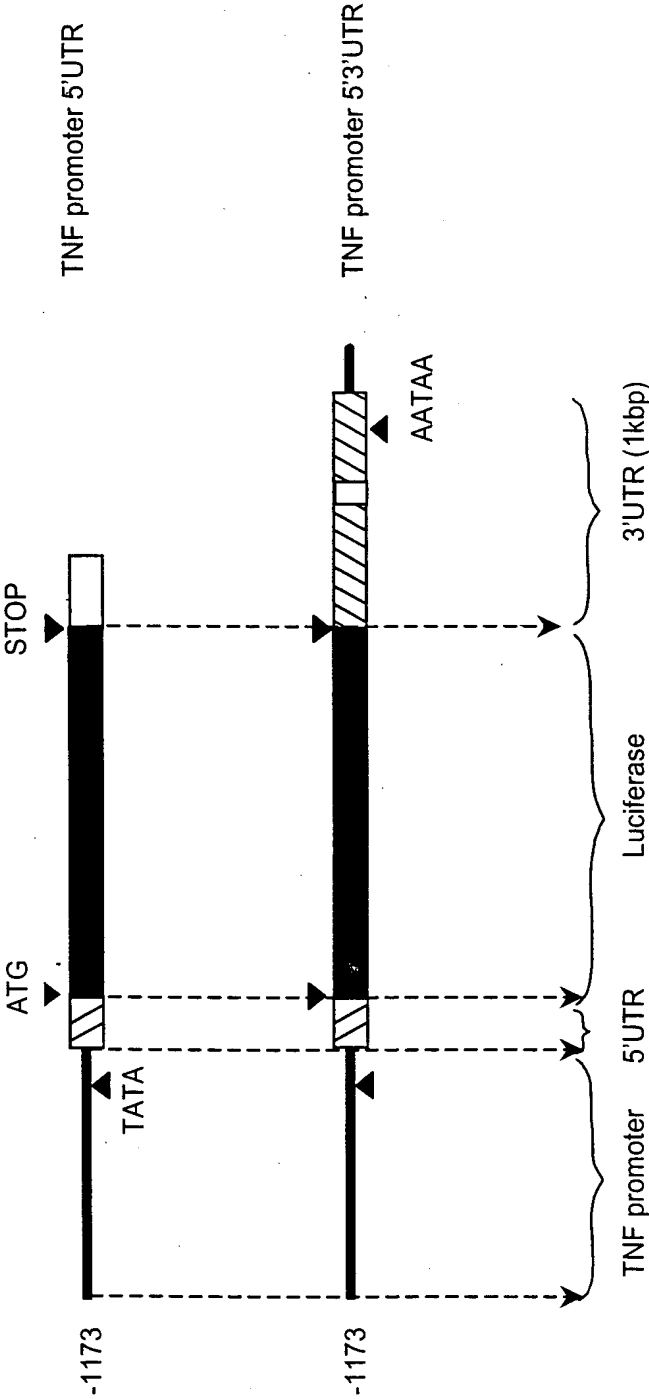
3061	agaagcttac	aactaaaaga	aactctaagg	ccaatttaaat	ccaagggttc	attctatgtg
3121	ctggagatgg	tgtacagtag	ggtgaggaaa	ccaaattctc	agttggcact	ggtgtaccct
3181	tgtacaggtg	atgtaacatc	tctgtgcctc	agtttgctca	ctataaaaata	gagacggtag
3241	gggtcatggt	gagcactacc	tgactagcat	ataagaagct	ttcagcaagt	gcagactact
3301	cttaccctact	tcccccaagc	acagttaggg	tgggggacag	ctgaagaggt	ggaaacatgt
3361	gcctgagaat	cctaatagaaa	tcggggtaaa	ggagcctgga	acacatcctg	tgaccccgcc
3421	tgtcctgtag	gaagccagtc	tctggaaagt	aaaatggaag	ggctgcttgg	gaactttgag
3481	gatatttagc	ccacccctc	atttttactt	ggggaaacta	aggcccagag	acctaagggtg
3541	actgcctaag	ttagcaagga	gaagtcttgg	gtattcatcc	caggttgggg	ggacccaatt
3601	attttctcaat	cccattgtat	tctggaatgg	gcaatttgct	cacgtcactg	tgacctagga
3661	acacgcgaat	gagaaccac	agctgagggc	ctctgcgcac	agaacagctg	ttctccccag
3721	gaaatcaact	ttttttaatt	gagaagctaa	aaaattattc	taagagaggt	agcccatcct
3781	aaaaatagct	gtaatgcaga	agttcatgtt	caaccaatca	tttttgctta	cgatgcaaaa
3841	attgaaaact	aagtttatta	gagagggttag	agaaggagga	gctctaagga	gaaaaaatcc
3901	tgtgccggga	aaccttgatt	gtggcttttt	aatgaatgaa	gaggcctccc	tgagcttaca
3961	atataaaaag	gggacagaga	ggtgaaggtc	tacacatcag	gggcttgctc	ttgcaaaacc
4021	aaaccacaag	acagacttgc	aaaagaaggc	atgcacagct	cagcactgct	ctggtgcctg
4081	gtcctcctga	ctgggggtgag	ggccagcca	ggccagggca	cccagtctga	gaacagctgc
4141	accacttcc	caggcaacct	gcctaacatg	cttcgagatc	t	

Claims

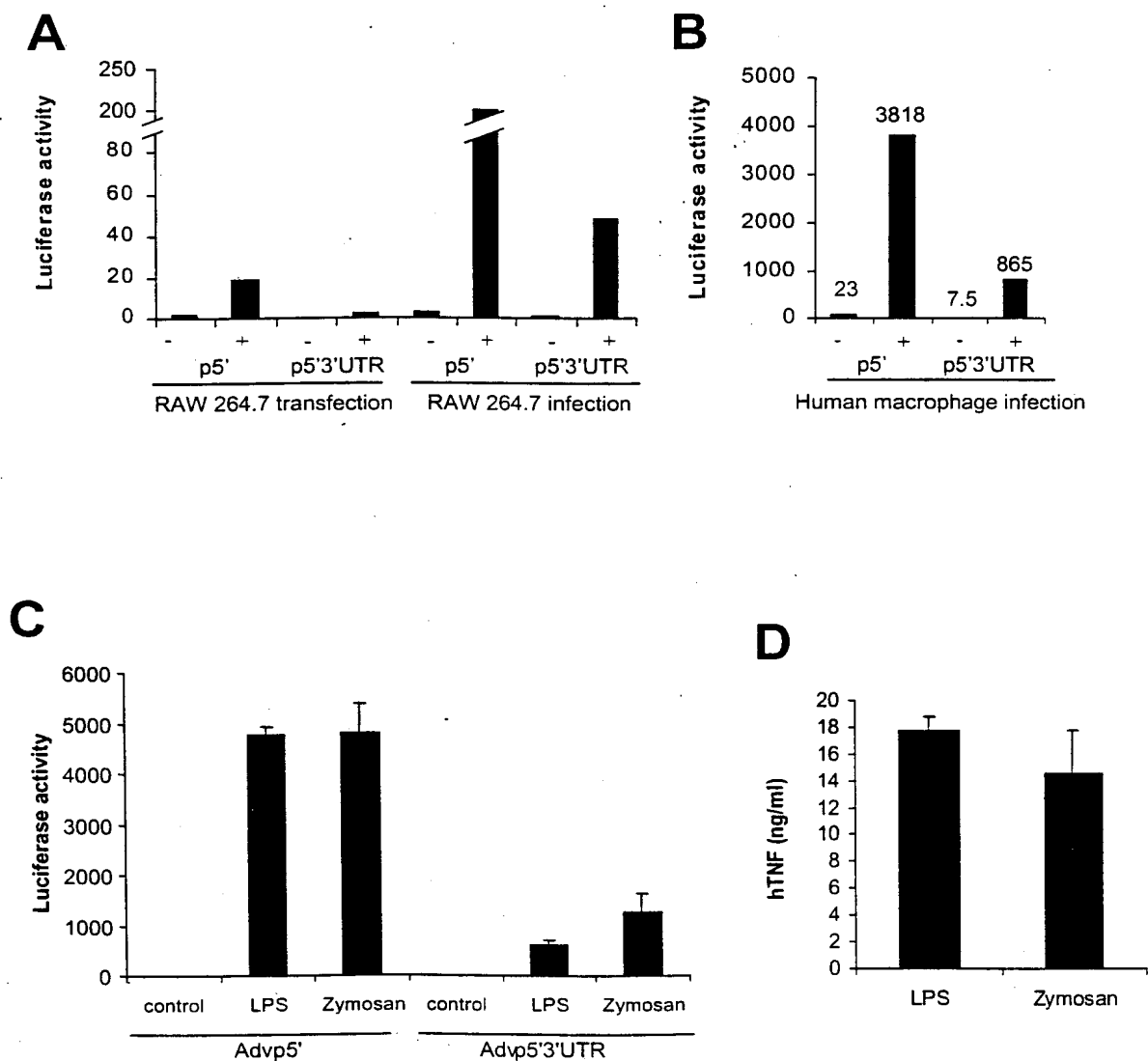
1. An adenoviral vector comprising:
 - (a) a mammalian regulatory region; and
 - (b) a reporter gene operatively linked to the mammalian regulatory region (a).
2. An adenoviral vector according to claim 1, wherein the mammalian regulatory region is a cytokine regulatory region.
3. An adenoviral vector according to claim 1 or claim 2, wherein the regulatory region is a promoter or enhancer.
4. An adenoviral vector according to claim 3, wherein the promoter is a TNF α promoter or an IL-10 promoter.
5. An adenoviral promoter according to claim 4, wherein the cytokine promoter is encompassed in -1173 to +130 of the human TNF gene.
6. An adenoviral vector according to any preceding claim, wherein the reporter gene is selected from luciferase, lacZ and chloramphenicol acetyltransferase (CAT).
7. An adenoviral vector according to any preceding claim additionally comprising a polyadenylation site operatively linked 3' to the reporter gene.
8. An adenoviral vector according to any preceding claim, additionally comprising 3' to the reporter gene a 3'-UTR (Untranslated Region).
9. A method of studying a mammalian regulatory region activity comprising inserting an adenoviral vector according to any preceding claim bearing the regulatory region into a cell and measuring the amount of reporter gene product produced in response to the regulatory region.

10. A method according to claim 9, additionally comprising exposing the adenoviral vector within the cell to one or more inhibitors or activators of the regulatory region.
11. A method according to claim 9 or claim 10, wherein the cell is selected from a monocytic cell, macrophage and a primary cell.
12. A method according to claim 11, wherein the primary cell is obtained from diseased tissue.
13. A method according to claim 12, wherein the diseased tissue is rheumatoid arthritic.
14. A cell comprising an adenoviral vector according to any one of claims 1 to 8.
15. A kit for use in a method according to claim 9, comprising an adenoviral vector according to any one of claims 1 to 8.
16. A method of screening a compound as an enhancer or an inhibitor of a mammalian regulatory region, comprising the use of an adenoviral vector according to any one of claims 1-8.

Figure 1

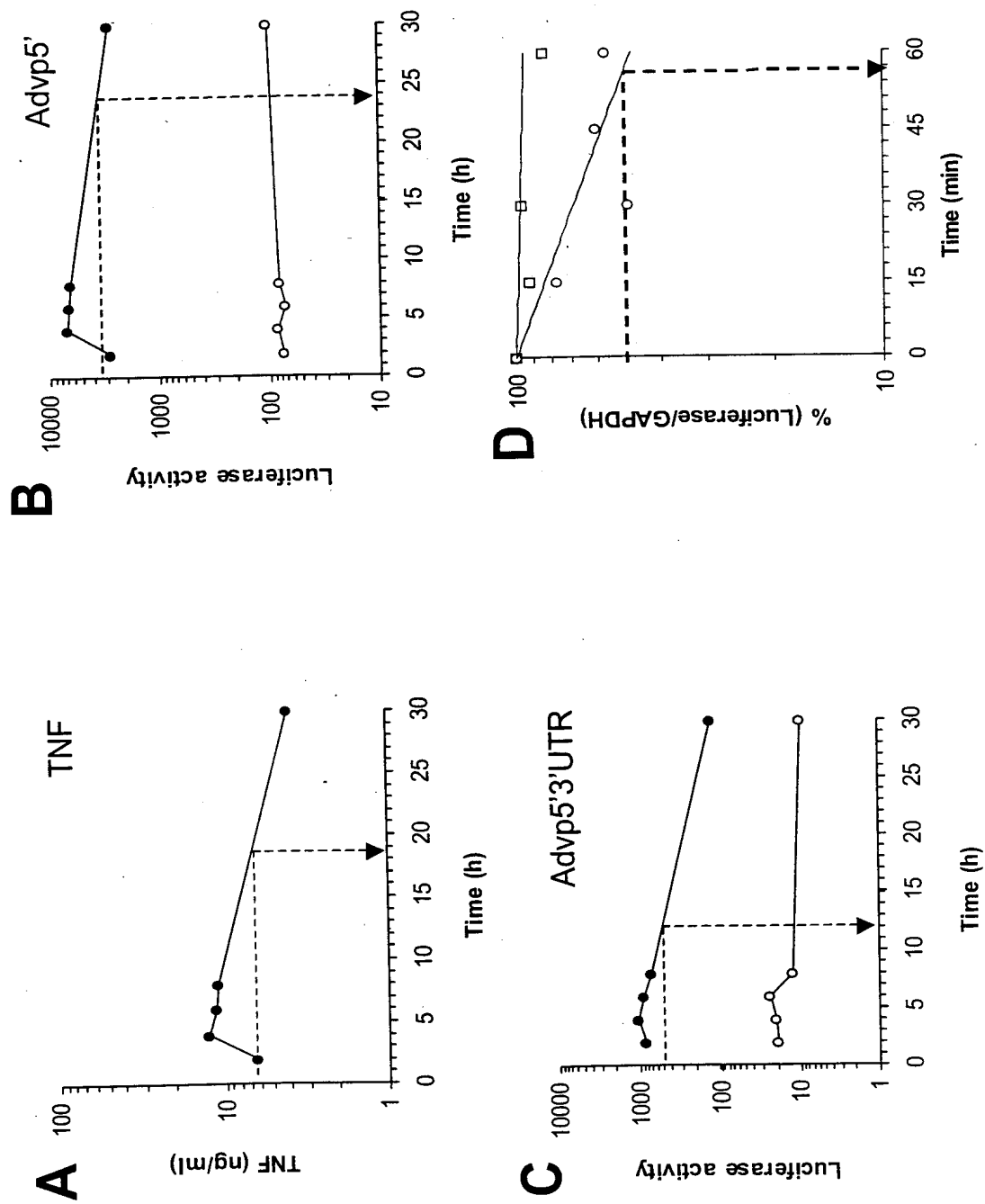


2/10

Figure 2

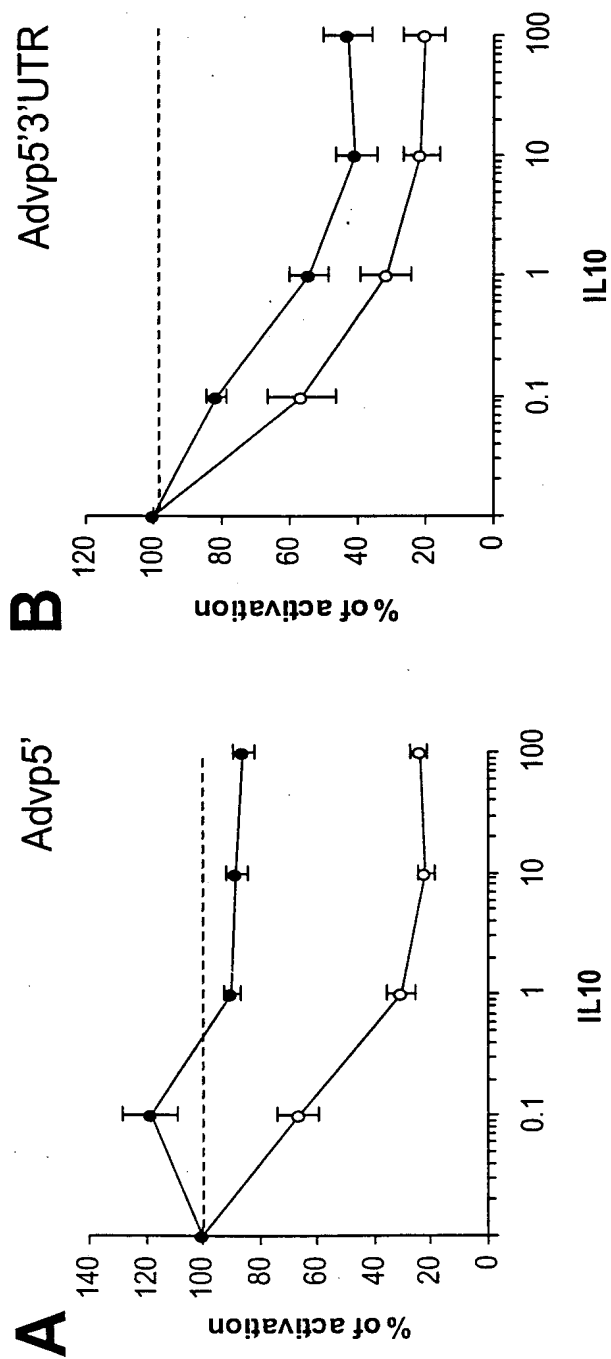
3/10

Figure 3



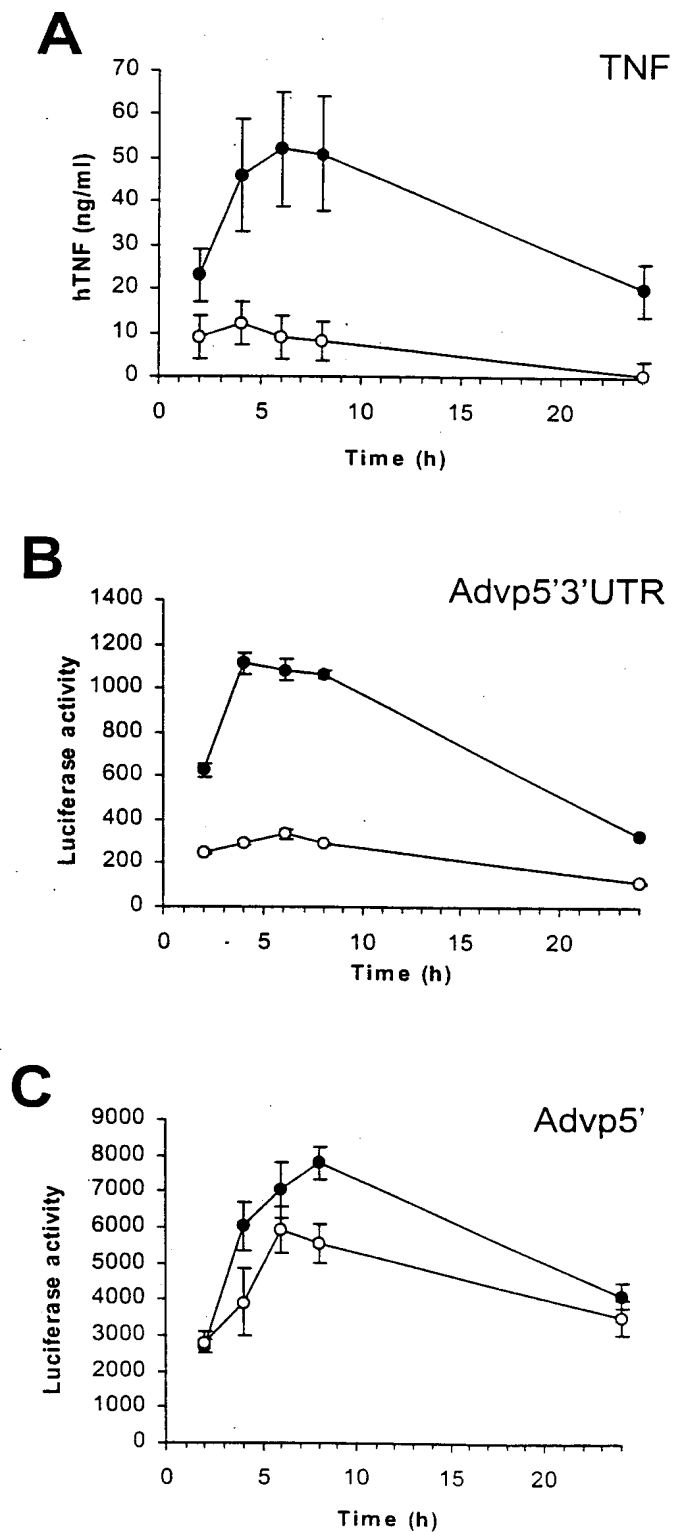
4/10

Figure 4



SUBSTITUTE SHEET (RULE 26)

5/10

Figure 5

6/10

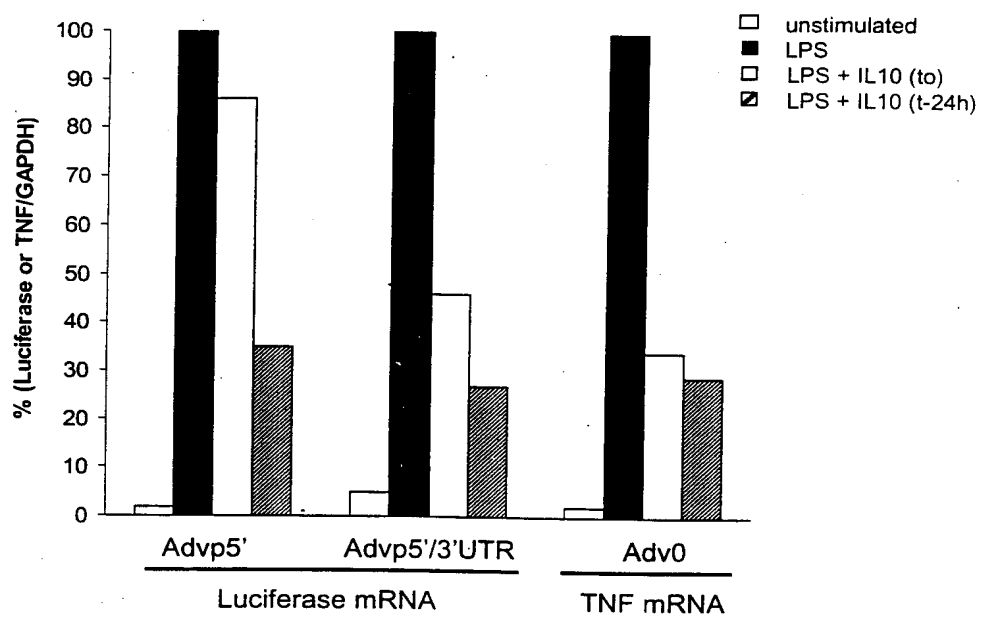
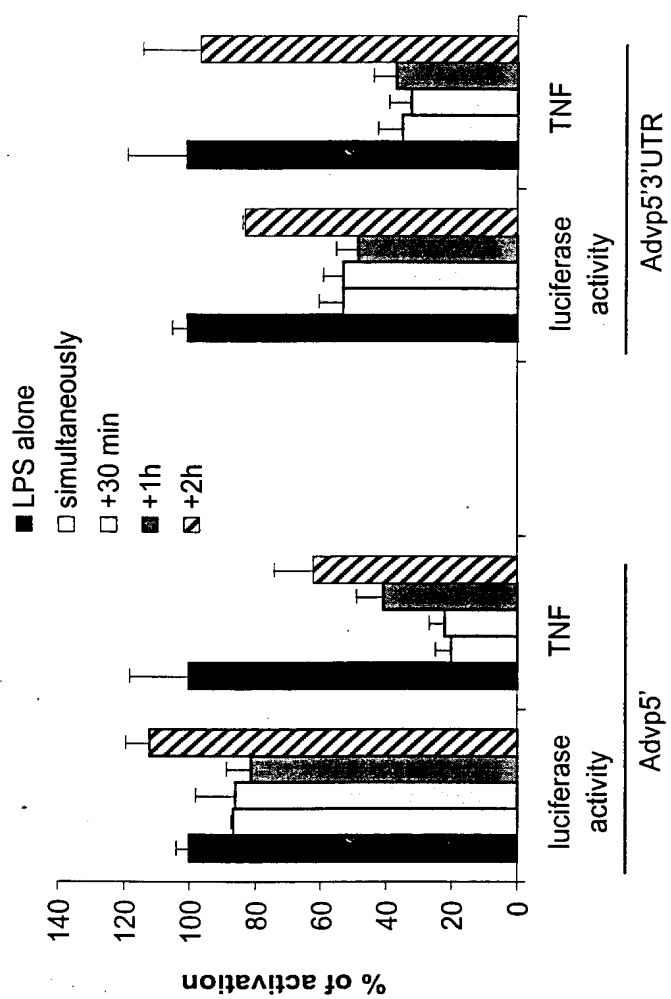
Figure 6

Figure 7



8/10

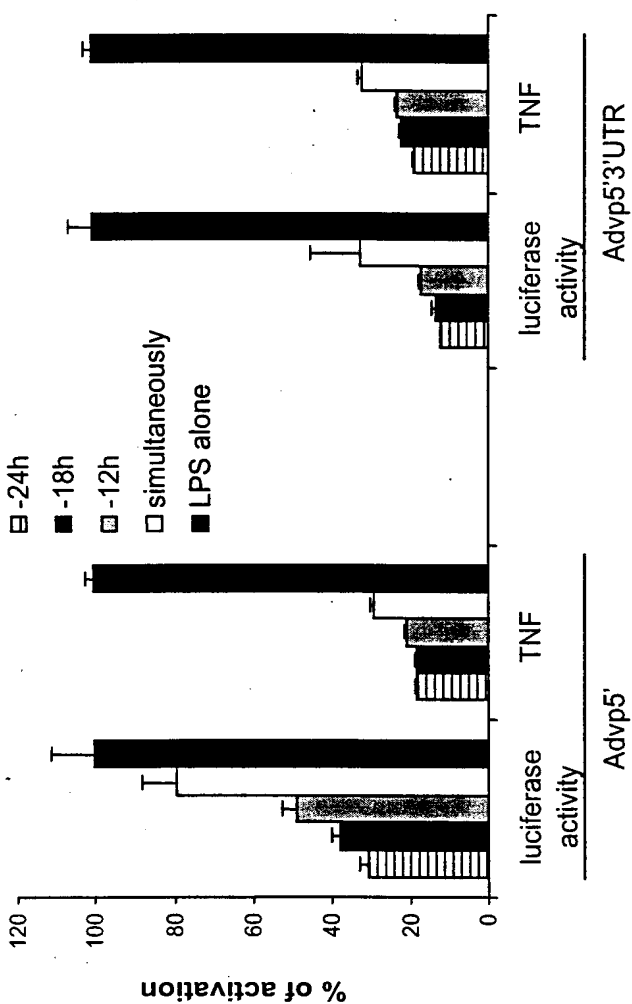


Figure 8

9/10

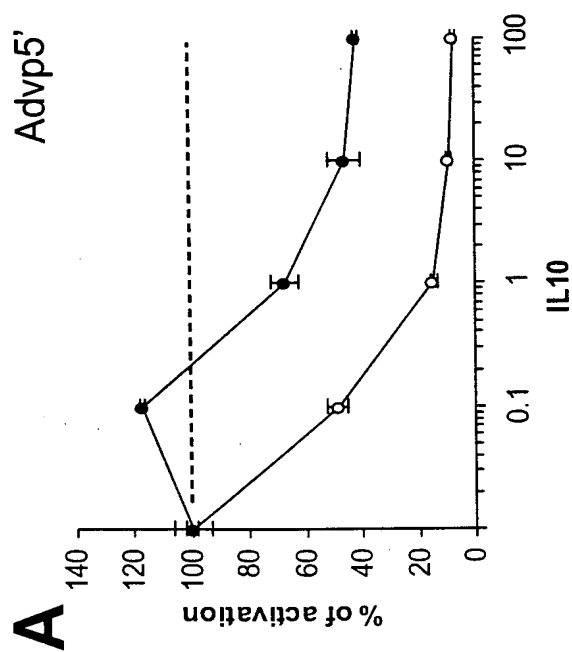
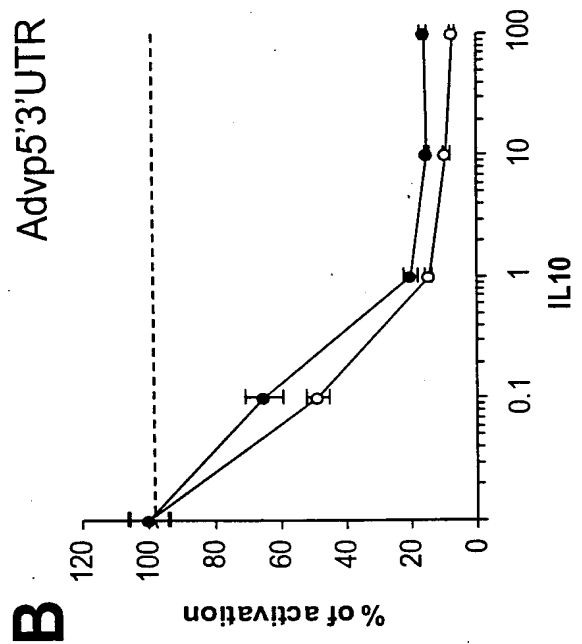
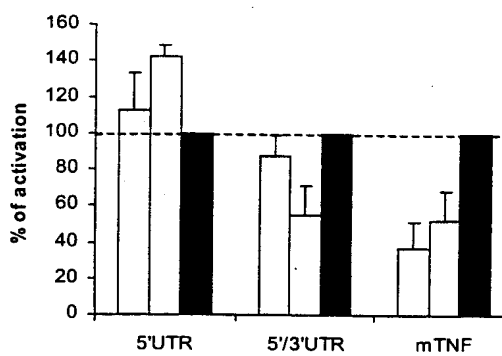


Figure 9

10/10

Figure 10

RAW 264.7 cells

Murine primary
peritoneal
macrophage